ANNALS OF SURGERY
Vol. 233, No. 4, 515–521


# Ex Vivo Sentinel Node Mapping in Carcinoma of the Colon and Rectum

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## Objective

Increasing evidence supports that the sentinel node (SN) is at greatest risk for harboring metastatic disease. This study describes a novel technique to identify the SN in colorectal carcinoma.

## Methods

Within 30 minutes of resection, colorectal specimens were injected submucosally with isosulfan blue in four quadrants. Blue lymphatic channels were identified in the mesentery and followed to the blue-stained SN(s), which were then harvested. The specimen was fixed in formalin and subsequently analyzed in the usual fashion. Blue-stained nodes that were negative by hematoxylin and eosin staining were further analyzed by immunohistochemical staining.

## Results

During a 6-month period, 26 patients with adenocarcinoma of the colon and/or rectum undergoing routine resection were studied. There were 18 men and 8 women ranging in age from 29 to 86 years (median 66). Blue-stained SNs were identified in 24 of 26 specimens. The mean number of SNs identified per patient was 2.8 ± 1.6. Seventy-three SNs were identified from a total of 479 lymph nodes harvested. The mean number of nodes identified per patient was 18.4 ± 7. A total of 67 lymph nodes in 12 patients were identified by hematoxylin and eosin staining to have evidence of metastatic disease. Fourteen (20%) of these nodes in six patients were stained blue. However, with immunohistochemical staining, only one blue node did not have evidence of metastatic tumor in a lymphatic basin with tumor present. Four patients (29%) whose lymphatic basins were negative by hematoxylin and eosin staining were upstaged by immunohistochemical staining of the SN.

## Conclusions

Ex vivo mapping of the colon and rectum is technically feasible and may provide a useful approach to the ultrastaging of colorectal carcinoma.

The principles of surgical resection of colorectal carcinoma dictate removal of the primary tumor, with adequate proximal and distal margins, and in continuity with the regional draining mesenteric lymphatics.[1] In addition to the curative intent, pathologic staging after surgical resection of the tumor is critical not only in establishing prognosis, but also in determining adjuvant treatment strategies.[2–4] The presence or absence of nodal metastases is the single most important prognostic factor in resectable carcinoma of the colon and rectum and currently is the primary indication for adjuvant systemic therapy.

In 1977, Cabanas[5] described a technique to identify drainage to a lymph node in penile carcinoma. Cabanas demonstrated that the first node draining the primary tumor, as defined by lymphangiography, was most likely to harbor metastatic disease. This node was referred to as the sentinel lymph node. In 1991, we described a novel intraoperative technique in a feline model to identify lymph nodes believed to be at greatest risk for harboring metastatic disease.[6] Based on the hypothesis that tumor cells metastasize in a defined anatomical pathway to a primary draining lymph node, intraoperative techniques were developed to identify and harvest the sentinel node (SN).[7] We called this approach selective lymphadenectomy. As a minimally invasive technique, sentinel lymphadenectomy can avoid the complications of a complete lymph node dissection in patients without evidence of metastatic disease while identifying patients with metastatic disease in the regional lymphatics who could potentially benefit from a therapeutic node dissection.

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Accepted for publication October 13, 2000.

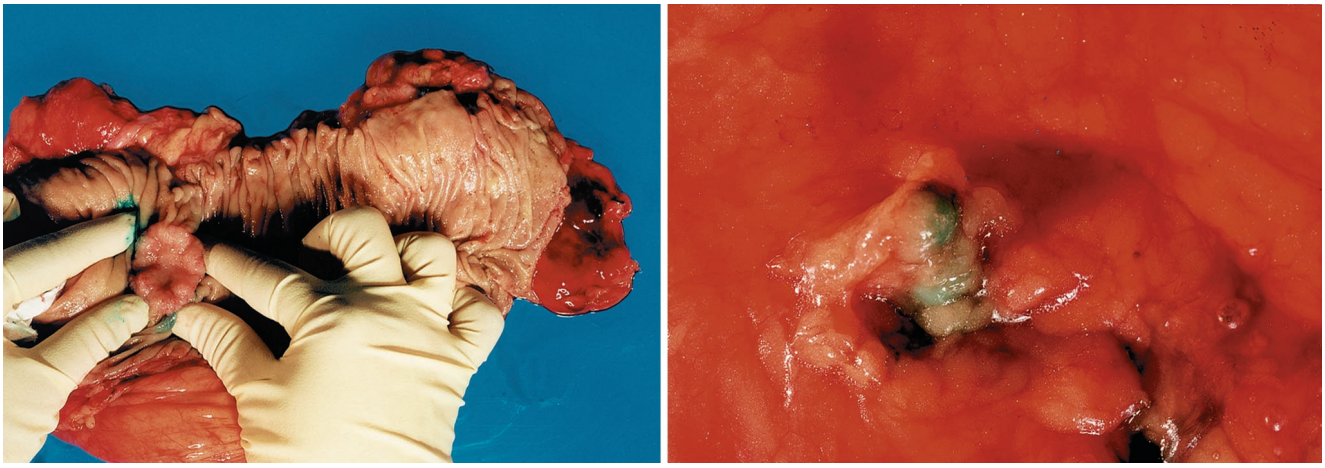

**Figure 1.** Technique of ex vivo mapping of the lymphatics. Lymphazurin (0.25 mL) is injected submucosally in four quadrants around the carcinoma (A). The specimen is gently massaged, and blue lymphatic channels are readily identified in its mesentery (B).

The feasibility of this approach has been shown in both cutaneous melanoma[7–10] and breast cancer.[11–15]

In addition to decreasing the complications from negative regional lymph node dissections, sentinel lymphadenectomy provides the opportunity to analyze a small volume of tissue more thoroughly, perhaps redefining our understanding of the biology of solid neoplasms. Routine pathologic analysis of the regional lymph nodes examines only a fraction of the total lymph node tissue submitted. However, the identification of the node that receives the primary drainage of the tumor, the SN, allows focused examination of a limited amount of tissue at greatest risk for harboring metastatic disease. Substantial evidence indicates that SN staging can upstage many patients with breast cancer.[16–19] The applicability of this approach in other solid neoplasms is being explored.[20]

Complications after resection of solid neoplasms of the colon and rectum are generally unrelated to the extent of the regional lymphadenectomy. For this reason, the advantage of minimizing the surgical intervention, as is done in cutaneous melanoma and breast cancer, has little value in these patients. In fact, if an SN dissection alone were performed, as is routinely done in cutaneous melanoma, this could prove to be problematic if the SNs, on further analysis, proved to have evidence of metastatic disease. However, the potential benefits of a more thorough examination of the regional lymphatics that more accurately stages the disease remain.[21] All previous attempts to identify the SN in solid tumors have used intraoperative techniques. This article describes a novel approach to identify the SN in carcinoma of the colon and rectum using ex vivo lymphatic mapping.

## METHODS

### Technique

Immediately after resection of the colon or rectum, specimens were delivered fresh to the Department of Pathology. After gross examination of the specimen and within 30 minutes of removal, the colon was incised longitudinally on the antimesenteric border (in the case of rectal tumors on the anterior border opposite the mesorectum). In opening the colon or rectum, no attempt was made to avoid the tumor if the tumor involved the antimesenteric wall of the colon. Using a tuberculin syringe, four separate submucosal injections of approximately 0.25 mL isosulfan blue (Lymphazurin 1% in aqueous solution; Ben Venue Labs, Bedford, OH) were performed in four quadrants around the tumor. The injections were placed at the proximal and distal margins of the tumor along the longitudinal axis of the specimen and at 90° from these injection sites. If the tumor extended to the antimesenteric segment of colon, an injection on both sides of the divided tumor was performed. A submucosal wheal of approximately 1 cm was obtained. The injection sites were then gently massaged for approximately 2 to 5 minutes (Fig. 1).

The mesentery was then examined by gently incising the overlying peritoneum at the base of the palpable tumor and at the junction of the mesentery with the colon. The mesenteric fat was bluntly separated, and using meticulous blunt dissection, blue lymphatic channels were identified and subsequently traced through the adipose tissue of the mesentery to a blue-stained lymph node. These blue-stained nodes were then individually harvested and submitted for histologic examination.

The rest of the resected specimen was then fixed in formalin overnight before further examination. Lymph nodes were harvested as follows: the peritoneum overlying the remainder of the mesentery was incised and then examined by careful palpation for the presence of lymph nodes. Any firm tissue remaining after gentle pressure on the mesenteric fat was isolated from the surrounding mesenteric fat and sent as a lymph node specimen for histologic examination by routine hematoxylin and eosin (H&E) staining.

## Pathologic Examination

Lymph nodes larger than 3 mm were bivalved in the longitudinal axis and embedded in paraffin. Lymph nodes smaller than 3 mm were embedded whole. A single section was routinely performed (two faces in bivalved lymph nodes and a single face in lymph nodes smaller than 3 mm). Histologic sections were processed in the usual manner, cut at 4 microns, and stained with H&E. Blue lymph nodes, when negative by routine H&E staining, were further analyzed.

Additional-level sections of paraffin-embedded blue lymph nodes were obtained at 300 $\mu$m. A single section was obtained after the first additional level and was stained with antibodies directed at low- and high-molecular-weight cytokeratin (Pan-Keratin AE1/3, CAM 5.2, 35bH11; prediluted, Vantana Medical Systems Inc., Tucson, Az). The immunostains were performed with avidin–biotin peroxidase using the BioTek Automated Staining System (Vantana Medical Systems Inc.). Formalin-fixed paraffin-embedded sections of tonsils were used as positive controls, and a section from each block was taken and incubated with negative control buffer (Vantana Medical Systems Inc.).

## Statistical Analysis

Diagnostic accuracy, sensitivity, and predictive value of a negative blue-stained node were calculated as described by Campbell and Machin.[22] Confidence intervals were computed utilizing Mathcad 2000 software (Math Soft Inc., Cambridge, MA) as described by Fleiss.[23]

# RESULTS

## Patient and Primary Tumor Characteristics

A total of 26 patients with colorectal cancer were studied. There were 18 men and 8 women ranging in age from 29 to 86 years (mean 65). Most patients had tumors in the sigmoid colon (14 patients). Six tumors were located in the right colon, three in the left colon, two in the rectum, and one in the transverse colon. There were 2 patients with T1 tumors, 5 patients with T2 tumors, 15 patients with T3 tumors, and 4 patients with T4 tumors. There were no patients with nonregional metastatic disease. The mean number of nodes identified was 18.4 (range 8–36).

## Identification of Blue-Stained Lymph Node

Identification of at least one blue-stained lymph node was successful 92% (95% confidence interval [CI] 73.4–95.3%) of the time, or in 24 of 26 specimens. In the remaining two patients, blue-stained tissue was harvested but this was not confirmed to be lymphoid tissue when examined histologically. The number of blue-stained lymph nodes removed ranged from 0 to 6, with a mean of 2.8 ± 1.5 blue nodes identified.

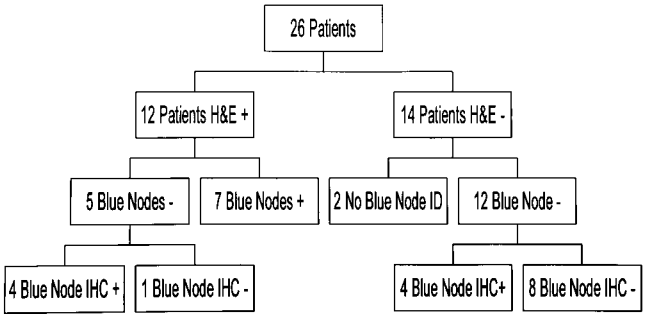


**Figure 2.** Distribution of patients undergoing ex vivo sentinel node mapping.

## H&E Analysis

A total of 479 histologically confirmed lymph nodes were harvested from the 26 patents. Sixty-seven lymph nodes in 12 patients were found to harbor metastatic disease by H&E staining. The number of positive lymph nodes ranged from 1 to 15 (mean 5.6). Figure 2 summarizes the results of the pathologic analysis of the regional lymph nodes. Tumor was present in blue-stained nodes harvested from seven patients. In five patients with metastatic disease, the blue-stained lymph nodes were negative by H&E staining (Table 1). The sensitivity of a blue-stained lymph node identifying metastatic disease with routine H&E staining was 58.3% (95% CI 28.6–77.2%). The negative predictive value was 73.6%.

## Pathologic Analysis by Step Sectioning and Immunohistochemical Staining

All patients with blue-stained lymph nodes that were negative by H&E staining underwent further analysis. Step sectioning was performed, and these sections were stained by H&E and for the presence of cytokeratin. Fourteen patients had no evidence of metastatic tumor by routine H&E staining. In two patients, deeper sections revealed evidence of metastatic disease by H&E staining, and in two other patients there was evidence of metastatic disease by immunohistochemical staining (Fig. 3). In five specimens in which the blue-stained node did not have metastatic disease on routine H&E staining, four were found to have evidence of metastatic disease. Only one patient who was found to have metastatic tumor on H&E staining did not have evidence of metastatic tumor in the blue-stained lymph node. Sensitivity of the blue-stained lymph node in identifying metastatic disease increased to 93.7% (95% CI 67.7–94.6%) over H&E examination alone. The predictive value of a negative blue-stained node was 92.8%.

# DISCUSSION

It is well recognized that substantial complications may be associated with radical lymphadenectomy of the axilla

**Table 1. PATHOLOGIC RESULTS: PRESENCE OF METASTATIC DISEASE IN REGIONAL LYMPH NODES**

| SN Analysis | H&E Analysis | | | IHC Analysis | | |
|---|---|---|---|---|---|---|
| | Present | Absent | Total | Present | Absent | Total |
| Positive | 7 | 0 | 7 | 15 | 0 | 15 |
| Negative | 5 | 14 | 19 | 1 | 10 | 11 |
| | 12 | 14 | 26 | 16 | 10 | 26 |

H&E, hematoxylin and eosin; IHC, immunohistochemistry; SN, sentinel node.
Sensitivity, H&E: 58.3% (95% CI 28.6–77.2).
Predictive value of a negative H&E test: 0.74.
Sensitivity, IHC: 93.7% (95% CI 67.7–94.6).
Predictive value of a negative IHC test: 0.93.

and groin. Lymphedema, the most significant long-term complication after radical inguinal or axillary node dissection, is not uncommon. For this reason, one of the major advantages of a minimally invasive SN dissection in patients with cutaneous melanoma and breast cancer is the potential to spare patients without nodal metastases an unnecessary complete lymph node dissection. In patients with breast cancer, sentinel lymphadenectomy has eliminated lymphedema and intercostal nerve paresthesia.[15]

The other potential major advantage of SN dissection is the potential to refine the staging of solid neoplasms.[16,18–20,24] Because there is little risk of increasing the risk of complications from a radical regional lymph node dissection in carcinoma of the colon or rectum, it would seem reasonable that the ability to study the primary draining lymph nodes in greater detail without intraoperative mapping techniques (as required in cutaneous melanoma and breast cancer) might provide a useful adjunct to the routine pathologic examination of the regional lymphatics.

This article describes an alternative approach to mapping the lymphatics and identifying the SN in resected carcinoma of the colon and rectum. While avoiding intraoperative manipulation of the specimen to identify the regional lymphatics, we were able to map the lymphatics of the colon and rectum and identify blue-stained lymph nodes that are consistent with the biologic definition of an SN. Ex vivo SN mapping provides a simple and potentially cost-effective technique to stage carcinoma of the colon and rectum without the need for intraoperative techniques.

Sentinel lymphadenectomy is generally accepted as the standard of surgical care in staging cutaneous melanoma.[25] However, its role remains investigational in breast cancer.[26] The potential utility of sentinel lymphadenectomy has now been described in several solid neoplasms, including squamous cell cancer of the vulva,[27–29] Merkel cell carcinoma,[30] thyroid cancer,[31] and most recently gastrointestinal malignancies.[20] All of these approaches use intraoperative lymphatic techniques to map the lymphatics and identify the SN

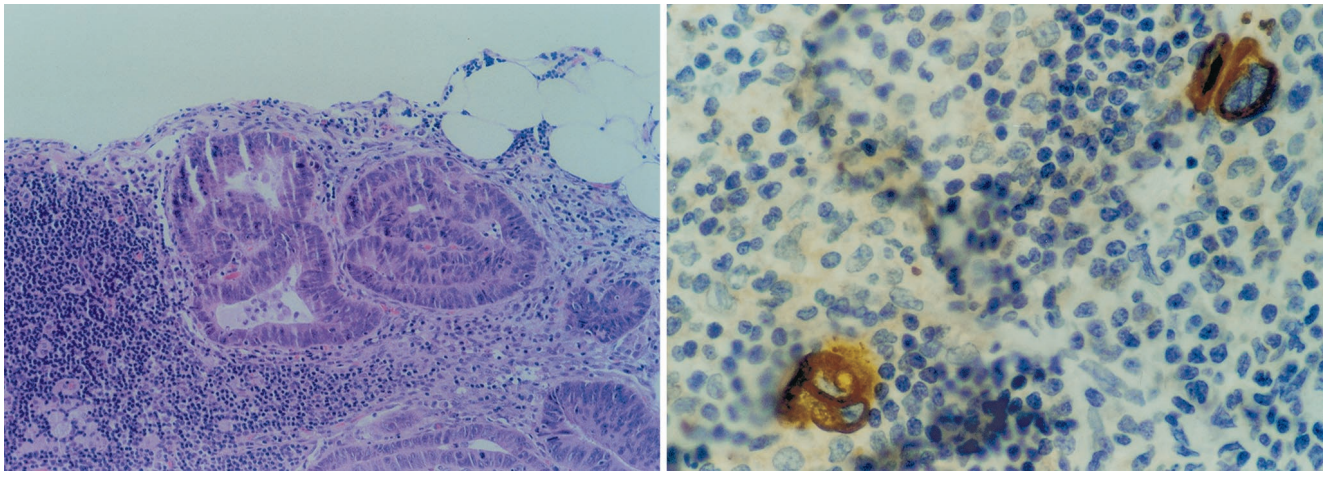

**Figure 3.** (A) Permanent sections obtained from deeper cuts showed tumor in the lymph node (×250, hematoxylin and eosin staining). (B) Using immunohistochemical staining for cytokeratin, single brown-staining cells, morphologically consistent with metastatic adenocarcinoma, are identified (×400, pancytokeratin stain).

at the time of surgery. However, surgical approaches could pose problems.

One of the vexing problems with intraoperative lymphatic mapping has been the issue of credentialing and the number of procedures necessary to be performed to demonstrate accurate harvesting of the SN.[32–35] The current American College of Surgeons Oncology Group trial evaluating sentinel lymphadenectomy requires a minimum of 30 procedures before being able to enroll patients.[36] The workload and practice patterns of general surgeons in the United States[37] indicate that approximately 13 colectomies are performed annually by general surgeons. The feasibility of learning intraoperative techniques in a timely fashion may be problematic except in high-volume settings. Our ex vivo approach may circumvent this issue because the pathology laboratory in a single institution should consolidate the case load of multiple general surgeons practicing in that institution, allowing the pathologists to gain expertise in lymphatic mapping.

Mechanical manipulation to increase lymphatic flow is commonly used to improve SN identification in both cutaneous melanoma[6,7] and breast cancer.[15] The potential for shedding tumor cells in this setting has been raised. Ex vivo SN mapping avoids this potential problem and allows the surgeon to perform an en bloc resection without fear of shedding tumor cells or disrupting the fascial planes that may be critical in minimizing local recurrences. Massaging the resected specimen to reproduce lymphatic flow in the in continuity lymphatic basin of the resected cancer specimen proved to be feasible and simple in the pathology laboratory. All but two patients had ready identification of at least one blue-stained lymph node.

For cutaneous malignancies in which avoidance of a complete lymph node dissection can substantially reduce sequelae, intraoperative techniques are critical to avoid unnecessary (histologically negative) lymphadenectomies. In contrast to cutaneous malignancies, the rationale for avoiding a complete lymphadenectomy is far less compelling in tumors of the gastrointestinal tract. In fact, the ability to return to perform a completion regional lymphadenectomy if the SN was found to have metastatic tumor, as is routinely recommended in cutaneous melanoma and breast cancer, is clearly more problematic. For this reason, identifying the SN at the time of surgery to avoid a lymphadenectomy is not critical.

At the initiation of this pilot study, we were unsure of the biologic character of the blue-stained nodes identified by ex vivo lymphatic mapping, but analysis showed that they are biologic SNs. In 12 patients who were found to have metastatic disease in the regional nodes, the blue-stained lymph node was found to be involved with metastatic tumor either by H&E staining or by immunohistochemistry in 11 patients. The blue-stained lymph node was the only node with metastatic tumor in five patients, four of which were positive only by immunohistochemistry. This ex vivo SN mapping technique identifies nodes that are consistent with the SN hypothesis.

A distinction must be made between harvesting increasing number of nodes and examining SNs. An SN represents the primary drainage of the primary tumor, and optimally all SNs should be examined to stage the disease most accurately. This is not equivalent to randomly examining a sample of nodes, which has been demonstrated to be inadequate when attempting to stage the regional lymphatics.[38] Because the SN is not necessarily the closest node geographically to the primary tumor, and because lymphatic drainage may vary from patient to patient, lymphatic mapping is critical to identify the SN accurately.

Immunohistochemistry can identify tumor deposits in approximately 20% of nodes that were normal by routine examination with H&E staining.[18] The addition of polymerase chain reaction technology identifies markers of tumor cells in 30% to 40% of H&E-negative lymph nodes.[21] Others have used immunohistochemistry to identify micrometastases in colorectal carcinoma.[39–42] In our population, 29% of patients who were negative by H&E staining were found to have small deposits of tumor by further analysis of the blue-stained lymph node using immunohistochemistry. One of these patients had only a T1 carcinoma.

The prognostic value of ultrastaging the regional lymph nodes in colorectal cancer remains controversial. Retrospective analysis has produced conflicting results. Liefers et al,[21] using a carcinoembryonic antigen-specific nested reverse transcriptase polymerase chain reaction (RT-PCR), observed a significant difference in survival in patients with stage II carcinoma of the colon and rectum in whom micrometastases were detected by this approach. Greenson et al[39] showed that immunoperoxidase techniques could identify micrometastatic disease, and the presence of micrometastatic disease correlated with a poorer prognosis. Other investigators, however, have failed to confirm that micrometastases, detected by immunohistochemical staining, significantly affect the prognosis.[40–42] The prognostic implications of our results have yet to be determined because the follow-up on our patient population is not sufficient to address this issue. However, it is intriguing that upstaging in 29% of our patients is consistent with the approximately 30% of patients with stage II disease in the Surveillance, Epidemiology, and End Results Program of the National Cancer Institute[43] who died of recurrent colorectal cancer at 10 years. Although it might seem that the natural next step would be to explore the defined benefit of adjuvant systemic therapy in the setting of micrometastatic disease identified by ultrastaging methods, we believe the biologic relevance of these findings needs to be validated in large multicenter studies, as is being proposed by the American College of Surgeons Oncology Group.

Although only one patient who was positive by H&E staining for metastatic tumor did not have a blue-stained lymph node with metastatic tumor, most histologically positive lymph nodes were not blue-stained (70%). Some have

suggested that the presence of macroscopic volumes of tumor might alter the lymphatic flow, which may explain this observation.[12] However, in contrast to cutaneous melanoma and breast cancer, in which the portal of entry into the lymphatic basin is being defined to identify patients who do not require a complete lymph node dissection, the major purpose of our approach is to refine our ability to stage colorectal carcinoma and redefine the biology of the disease. Patients who are found by routine H&E staining of the regional lymph nodes to have metastatic disease do not require further analysis. However, our data suggest that in H&E-negative lymphatic basins, a more detailed analysis of the SN can upstage the disease in a significant number of patients. Our approach is not designed to replace routine pathologic analysis but rather to serve as an adjunct in patients found to be node-negative by routine H&E staining.

Two patients on further sectioning were identified as having metastatic disease by H&E staining alone. It is well recognized that serial sectioning in breast cancer can upstage disease in patients with apparently node-negative disease.[44] The International Breast Cancer Study Group upstaged disease in 9% of 921 breast cancer patients using serial sectioning. However, it is unknown whether this approach in colorectal cancer would have similar yields, and the time, effort, and cost necessary to study nonselectively all regional nodes in this fashion are generally considered prohibitive.

Several methods are available to identify micrometastatic disease. Molecular detection of micrometastases using RT-PCR requires snap freezing of the lymph node in liquid nitrogen for isolation of RNA. Although molecular detection can identify a single tumor cell in a background of 1 to 10 million normal cells, because of the rapid degradation of RNA, this approach may not be feasible except in specialized laboratories. In addition, normal epithelial cells may be detected by RT-PCR,[45] which may compromise the clinical utility of this approach. Currently, it is estimated that the cost of RT-PCR analysis of a single SN approaches $200 (personal communication, Reintgen DS); this does not include the extra handling and storage of specimens. We estimate that the cost of processing a single blue-stained node in our laboratory, including the extra handling of the specimen in the anatomical pathology laboratory, to be approximately $55, a substantial savings over analysis by RT-PCR. Others have used immunohistochemical staining of all regional nodes to identify micrometastases. However, immunohistochemical staining of the entire lymph node basin, in which on average 15 nodes need to be examined,[46] would be excessively expensive and time-consuming. For this reason, a simple technique, using readily available reagents, to identify the nodes at greatest risk for upstaging carcinoma of the colon and rectum has great appeal. The approach described in this article was easily taught to pathology prosectors and proved to be a time-efficient technique that can be easily performed to analyze more thoroughly small volumes of tissue at apparently increased risk for harboring metastatic disease. We intend to continue analysis of this approach to determine the prognostic significance and clinical relevance of ultrastaging by ex vivo lymphatic mapping in patients with carcinoma of the colon and rectum.

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